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14. ABSTRACT The long term objective of this work is to elucidate metabolic pathways which can be used to reduce the need for radical surgery in patients at high risk for prostate cancer or with early stage disease. The hypothesis to be tested is that alterations to lipoxygenase (LOX) and cyclooxygenase (COX) activity in early prostate cancer represent distinct druggable pathways which can be treated in conjunction with the PPAR γ signaling pathway to slow or prevent the development and progression of prostate cancer. In the second year of funding, we have generated and applied the various viral vectors (PPAR γ siRNAs, COX and LOX shRNA and overexpression) and have generated many of the tissue recombinants needed to perform the proposed experiments. We have completed the majority of the experiments proposed in specific aim 1 and are writing this work up for publication. As in the mouse model loss of PPAR γ function in human epithelium leads to a PIN phenotype which can be promoted to cancer with additional genetic insults. We have generated cells and recombinants with altered COX and LOX expression for the experiments proposed in specific aim 2 which are now ongoing. Work for specific aim 3 is just starting. The second year of work has demonstrated that the combination of PPAR γ loss with other common genetic insults can cause progression of a PIN phenotype.					
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Annual Report

PCRP Idea Development Award

W81XWH-07-1-0479

Modulation of PPAR-Gamma Signaling in Prostatic Carcinogenesis

P.I. Simon W. Hayward, PhD

Introduction

This project examines the relationship between PPAR γ and carcinogenesis. PPAR γ sits at a critical juncture in cellular differentiation and metabolism being involved in both differentiation and in the regulation of stress responses mediated through the cyclooxygenase (COX) and lipoxygenase (LOX) pathways of fatty acid metabolism. The basis for this project was the observation that in human prostate cancer there is an early loss of enzymes responsible for the production of the putative endogenous ligands for PPAR γ , presumed to result in a decrease in receptor function. We have found that loss of PPAR γ function can result in the generation of premalignant prostatic lesions in mice (Jiang et al 2009). We have also shown that there is an associated upregulation of COX pathways which would generate increases in prostaglandin production and oxidative stress, which could underlie such a pathology. This project sets out to examine interactions between the PPAR γ , COX and LOX pathways and their role in carcinogenesis. We are using predominantly tissue recombination models involving human prostatic epithelial cells. The use of human cells is important in that there are significant differences between the fatty acid metabolic pathways between humans and mice.

Body

Status of progress in relation to the original SOW is summarized below:

Task 1. Examine the in vivo consequences of suppression of PPAR γ signaling in human prostatic epithelium.

Generate and test PPRE-luciferase reporter construct (months 1-3) **Completed.**

Generate and test viral vectors carrying siRNA targeting human PPAR γ 1 and γ 2 (months 1-4) **Completed.**

Grow up and infect hPrE and hPrEshp16 cells using PPAR γ siRNA and PPRE-luc viral particles. Select infected cells (months 4-8). **Completed siRNA phase – some modifications to specific cells used noted below.**

Validate function of viral constructs in cells (Western blot and luciferase assay – qRT-PCR can also be used for the PPRE-luc is PPAR γ 1 suppression is unexpectedly efficient) (months 5-9) **Completed.**

Generate and graft tissue recombinants using infected epithelial cells and NPF and CAF (or rUGM controls) (months 6-10) **Completed.**

Harvest recombinants, process for biochemical and immunohistochemical analysis (months 10-17) **Completed.**

Histopathologic assessment of slides (months 15-20). **Completed.**

Generation and analysis of proliferative and apoptotic indices (months 15-20) **Ongoing.**

Summation of data set and manuscript preparation (months 20-24) **Ongoing.**

Task 2. Examine the in vitro and in vivo consequences of overexpression of cyclooxygenase –1 or –2 or 15-lipoxygenase-1 in human prostatic epithelium.

Generate and test viral vectors carrying expression constructs for COX-1, COX-2 and 15-LOX-1 (months 2-5). **Completed.**

Grow up and infect hPrE and hPrEshp16 cells using PPRE-luc viral particles in combination with COX-1, -2 or 15-LOX-1 overexpression viral vectors. Select infected cells (months 5-9). **Completed.**

Validate function of viral constructs in cells (Western blot and luciferase assay) (months 5-10) **Completed in modified form, as noted below.**

Generate and graft tissue recombinants using infected epithelial cells and NPF rUGM (months 6-10) **Ongoing.**

Harvest recombinants, process for biochemical and immunohistochemical analysis (months 10-17) **Ongoing.**

Histopathologic assessment of slides (months 15-20) **Ongoing.**

Generation and analysis of proliferative and apoptotic indices (months 15-20) **Ongoing.**

Summation of data set and manuscript preparation (months 20-24) **Not yet started.**

Task 3. Examine protective effects of PPAR γ agonists and/or COX/LOX inhibitors against the neogenesis of PIN or progression of prostate cancer.

Ongoing - see note below regarding tissue collection for this task.

Summary of Activity

We have been pursuing the project as outlined in the statement of work, as noted above. In general, the work is proceeding as planned and on time. The mouse work, which was the basis for this project, although not strictly a part of it, is completed and will be published in October 2009 (advanced electronic publication appended). Confirmation that the same premalignant changes and progression seen in murine cells also occur in human epithelial cells has been generated using siRNA knockdown of PPAR γ -1/-2 in human prostatic epithelial cell lines NHPe1 and BHPe1, NHPe1-C-MYC- and NHPe0-SV40Tag/hTERT followed by tissue recombination with prostate-inductive rat urogenital sinus mesenchyme (rUGM).

As described in the first annual report we have generated new human prostatic epithelial cell lines which better recapitulate human prostatic biology than those that were available at the time of submission. A descriptive manuscript is currently in revision in Stem Cells. Consistent with the first task in the statement of work we have shown that PPAR γ suppression in these cells results in the formation of a low grade PIN phenotype.

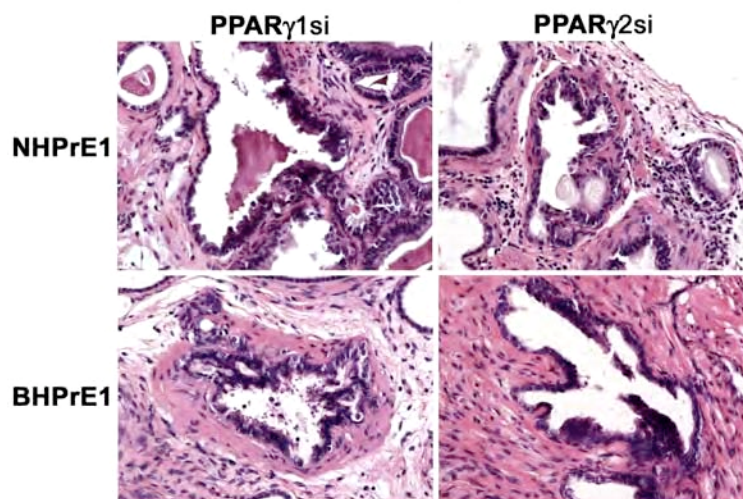


Figure 1. Tissue recombinants made by NHPe1 or BHPe1-PPAR γ 1 or γ 2 siRNA with rat UGM post-grafting for three months under the subrenal capsule of SCID mice showed human low grade PIN pathogenesis.

In order to further develop this model we have combined PPAR γ suppression with other genetic insults. Specifically we have used combinations of PPAR γ suppression along with c-Myc activation or SV40T expression. The findings of these studies, which are currently being written up for publication, are that addition of c-myc to PPAR γ suppression gives rise to a high grade PIN phenotype with some foci of invasion, while the use of PPAR γ suppression in a human prostatic epithelial cell line immortalized with SV40T gives rise to high grade PIN and local invasive cancer. Due to the relatively uncontrolled nature of SV40T action we are limiting pursuit of this model, but will pursue the PPAR γ /myc combination. As

noted in the first annual report we initially proposed to use epithelial cells in which p16 was knocked down as a second genetic insult and we have been exploring these other options as an alternative.

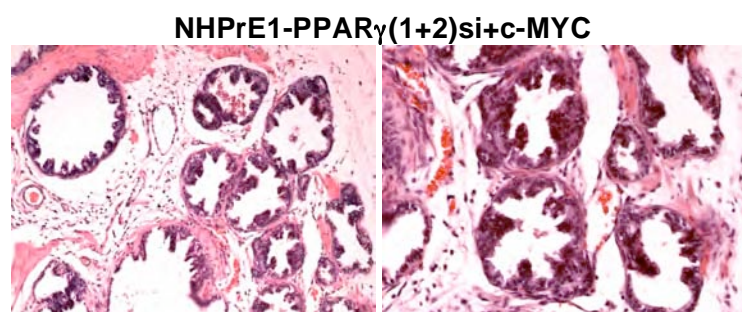


Figure 2. Tissue recombinants made using NHPrE1-PPAR γ (1+2) siRNA with rat UGM at three months post-grafting under the renal capsule of SCID mice showing human high grade PIN formation. *Left*, low mag.; *Right*, high mag.

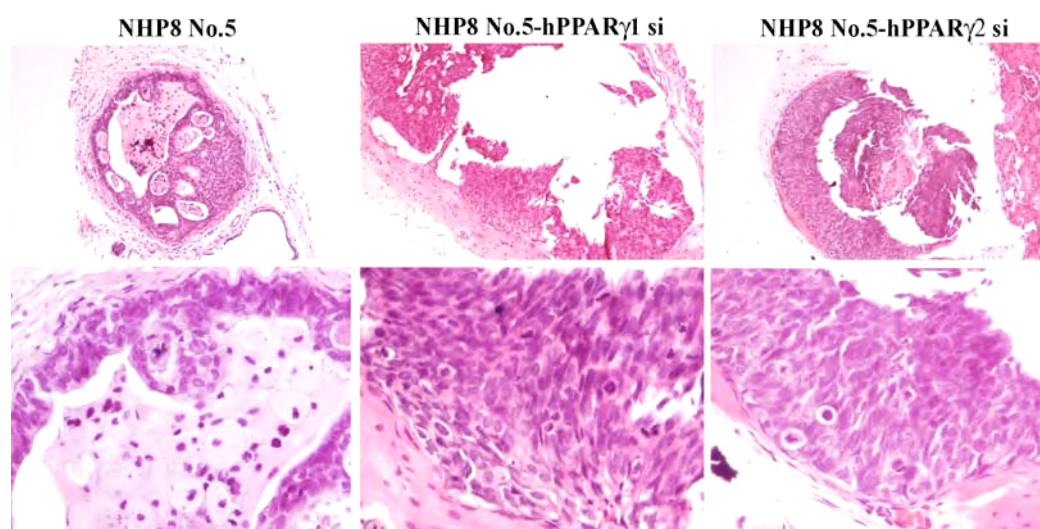


Figure 3. Tissue recombinants made by NHP8 No. 5 cells (originated from primary NHPrE0 cells retrovirally immortalized by SV40Tag and hTERT)-PPAR γ 1 or - γ 2 siRNA with rat UGM post-grafting for three months under the subrenal capsule of SCID mice showed human high grade PIN formation and local invasion compared to those glandular structure by the control cells. *Upper*, low mag.; *Low*, high mag.

In the first annual report we described the possible use of PTEN and PPAR γ co-suppression, using a tetracycline regulated PTEN suppression construct. However unexpected cross talk between tetracycline activity and the PPAR γ pathway has made data generated in this way challenging to interpret. While these results are interesting they are probably not pertinent to the main thrust of this proposal and as such are no longer being actively pursued.

In task 2 we have continued studies in which the expression of lipoxxygenase and cyclooxygenase is regulated (expression or suppression). We have now generated COX-1 and -2, 15-LOX-1 overexpressing cells which have all been validated and recombinants have been generated as proposed.

These experiments are currently in mice awaiting harvest. 15-LOX-2 suppression experiments are slightly more advanced but the data have not yet been analyzed.

As described in the first annual report the movement toward laparoscopic/robotic prostatectomy has had a negative impact on the quality of tissue available for research, required for task 3. We have worked with the VICC Tissue Acquisition Core to sidestep this problem and with some of the urologic surgeons to modify their technique – allowing the tissue to be removed from the body cavity immediately after resection, rather than at the end of the procedure. This limits the time that the tissue is kept warm but with no blood supply. This modification, while it is slowing down tissue collection does seem to be improving recovery of tumor.

Key Research Accomplishments

- Completed characterization of mice with conditional knockout of PPAR γ in the prostate. Manuscript will be published in the Oct. 16th issue of Cell Death and Differentiation (reference cited in reportable outcomes section).
- Analysis of the new human prostatic epithelial cell lines (BHPRE1, and NHPRE1) described in the first annual report has been completed. A descriptive manuscript is presently in revision following review by the journal Stem Cells.
- Generated knockdown of PPAR γ -1/-2 by siRNA in human prostatic epithelial cells. Demonstrated that in tissue recombination models these undergo similar profiles of phenotypic changes to those seen in mouse prostate in which expression of this gene is suppressed, notably with the consistent expression of a PIN phenotype.
- Generated cells in which both PPAR γ was suppressed while c-Myc expression was elevated or SV40Tag/hTERT were expressed. Demonstrated that these cells give rise to high grade PIN with some invasive foci in a tissue recombination assay.
- Generated the cellular modifications necessary for task 2.

Reportable Outcomes

Disruption of PPAR γ signaling results in mouse prostatic intraepithelial neoplasia involving active autophagy. M Jiang, S Fernandez, W G Jerome, Y He, X Yu, H Cai, B Boone, Y Yi, M A Magnuson, P Roy-Burman, R J Matusik, S B Shappell and S W Hayward Cell Death Differ advance online publication, October 16, 2009; doi:10.1038/cdd.2009.148 (copy appended)

Conclusions

This work is proceeding along the lines proposed, we have had some tissue collection delays but do not anticipate that these should be problematic, however this situation will be monitored and we have alternative models available as needed that can be used to test the central concept of task 3. Improvements in the cell lines available to us have allowed for improvements in the overall model system, and have allowed us to expand the range of mutations which we can examine. Some minor technical problems – for example the unexpected interactions between PPAR γ and tetracycline have cropped up but these should not interfere with the completion of the work in a timely manner.

Disruption of PPAR γ signaling results in mouse prostatic intraepithelial neoplasia involving active autophagy

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Peroxisome proliferator-activated receptor-gamma (PPAR γ) regulates the interface between cellular lipid metabolism, redox status and organelle differentiation. Conditional prostatic epithelial knockout of PPAR γ in mice resulted in focal hyperplasia which developed into mouse prostatic intraepithelial neoplasia (mPIN). The grade of PIN became more severe with time. Electron microscopy (EM) showed accumulated secondary lysosomes containing cellular organelles and debris suggestive of autophagy. Consistent with this analysis the autophagy marker LC-3 was found to be upregulated in areas of PIN in PPAR γ KO tissues. We selectively knocked down PPAR γ 2 isoform in wild-type mouse prostatic epithelial cells and examined the consequences of this in a tissue recombination model. Histopathologically grafted tissues resembled the conditional PPAR γ KO mouse prostates. EM studies of PPAR γ - and PPAR γ 2-deficient epithelial cells *in vitro* were suggestive of autophagy, consistent with the prostatic tissue analysis. This was confirmed by examining expression of beclin-1 and LC-3. Gene expression profiling in PPAR γ -/ γ 2-deficient cells indicated a major dysregulation of cell cycle control and metabolic signaling networks related to peroxisomal and lysosomal maturation, lipid oxidation and degradation. The putative autophagic phenotypes of PPAR γ -deficient cells could be rescued by re-expression of either γ 1 or γ 2 isoform. We conclude that disruption of PPAR γ signaling results in autophagy and oxidative stress during mPIN pathogenesis.

Cell Death and Differentiation advance online publication, 16 October 2009; doi:10.1038/cdd.2009.148

Peroxisome proliferator-activated receptors (PPARs) regulate gene transcription in response to peroxisome proliferators, fatty acids, and other endogenous ligands.^{1,2} There are two isoforms of PPAR γ denoted as PPAR γ 1 and PPAR γ 2. These represent proteins derived from alternative start sites in the same gene giving rise to two isoforms, with PPAR γ 2 having an earlier start site with an additional 30 amino acid residues.³ PPAR γ regulates differentiation, proliferation and apoptosis in both stromal⁴ and parenchymal epithelial cells,^{5–8} involving in many signaling pathways during the pathogenesis of inflammation and oxidative stress.^{9,10} The links between PPAR γ target genes and the relevance of alterations of PPAR γ functions to human pathology are beginning to be elucidated.¹¹

Many prostate cancer (Pca) patients with early disease could potentially be treated conservatively. Tissue-based and *in vitro* studies suggest that alterations in PPAR γ activity may be involved in Pca and that PPAR γ may be a candidate target for Pca therapy.^{12–14}

Recent studies have reported autophagy in prostate cancer cell lines.^{15–17} To gain insights into whether autophagy is involved in the processes of dysregulated lipid metabolism and induced oxidative stress accompanying the prostate proliferative epithelial lesions, we characterized the PPAR γ -deficient mouse prostate models. Autophagy accompanied the altered cellular proliferation and differentiation that resulted from PPAR γ -deficiency in mouse prostate models. This paper examines the links between PPAR γ activity and the subcellular and histopathologic changes taking place in

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Keywords: PPAR γ ; Peroxisome proliferator-activated receptor gamma (γ); Autophagy; Oxidative Stress; Differentiation

Abbreviations: Pca, prostate cancer or prostate carcinoma; PIN, prostatic intraepithelial neoplasia; PPRe, peroxisome proliferator response element; mPre, a non-malignant mouse prostate epithelial cell line spontaneously immortalized from an adult wild-type C57Bl6 mouse; mPre-PPAR γ KO (mPre- γ KO), a PPAR γ knockout mouse prostate epithelial cell line spontaneously immortalized from an adult PBCre^{tg}/PPAR γ ^{fllox/fllox} mouse prostate; mPre-pSIR-PPAR γ 2 shRNA (mPre- γ 2sh), a PPAR γ 2 knockdown mouse prostate epithelial cell line transfected by U6-mouse PPAR γ 2 shRNA in pSIR retroviral vector into mPre cells; mPre-pSIR-empty vector (mPre-pSIR), a control pSIR vector-transfected mouse prostate epithelial cell line; mPre-PPAR γ KO-PPAR γ 1 wild-type (WT) (mPre- γ KO- γ 1WT), mPre- γ KO overexpressing mouse PPAR γ 1 WT full-length cDNA; mPre-PPAR γ KO-PPAR γ 2 wild-type (WT) (mPre- γ KO- γ 2WT), mPre- γ KO overexpressing mouse PPAR γ 2 WT full-length cDNA; mPre-PPAR γ KO-empty vector (EV) (mPre- γ KO-EV) transduction control, mPre- γ KO retrovirally transduced using a control empty vector; UGS, urogenital sinus; UGM, urogenital mesenchymal cell; KO, knockout; KD, knockdown

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the murine prostate. Details of these changes were examined using parallel *in vitro* models.

Results

Progressive mPIN in PBCre4^{tg/0}/PPAR γ ^{flox/flox} transgenic mice. We generated PBCre4^{tg/0}/PPAR γ ^{flox/flox} double transgenic mice on a C57/Bl6 background. A PBCre4^{tg/0}/PPAR γ ^{flox/flox} mouse line was selected which strongly excised exon 2 of PPAR γ (both γ 1 and γ 2 isoforms) in the anterior (AP) and ventral prostate (VP) with weaker excision in the lateral (LP) and dorsal (DP) prostatic lobes. Loss of prostatic PPAR γ protein expression was confirmed using immunohistochemistry (Supplementary Information S1a).

The prostatic phenotypes of mice carrying two *flox* alleles but no Cre were indistinguishable from their WT counterparts. Beginning at three months of age we observed histologic alterations indicative of mPIN in the KO mice.^{18,19} Epithelial hyperplasia with mild cytologic atypia and local inflammatory cell accumulation was observed in the AP and VP (Figure 1a), but not in the DP and LP. The involved lobes showed epithelial stratification, consistent with increased cell proliferation. Nuclei were enlarged, with mild hyperchromasia and focally more prominent nucleoli, compatible with lesions previously categorized as mouse prostatic intraepithelial neoplasia (PIN) I in genetically engineered mouse (GEM) models¹⁹ here designated low grade PIN (LGPIN).

Older KO mice showed progressively more widespread prostatic epithelial hyperplasia with increased nuclear atypia in AP and VP, satisfying key criteria for NCI MMHCC mPIN.¹⁸ These progressive changes were similar to lesions designated as PIN II and III¹⁹ (Figures 1b-c) here designated high grade PIN (HGPIN). Cytoplasm was increasingly amphophilic, suggesting decreased secretory differentiation. Nuclear enlargement with chromatin clumping and prominent nucleoli was noted (Figure 1). No foci of invasive carcinoma were identified in PPAR γ KO mice up to 15 months of age.

No significant gross differences were seen between prostatic lobe weights from 12 month old KO and WT mice (Supplementary Information S1b). In the AP, decreased branching and associated expansion of some individual AP ducts were noted in KO versus control mice, first discernible in the 7-12 month age group (Figure 1d).

PAS staining illustrated decreased luminal secretions in the conditional PPAR γ KO mouse prostatic epithelia (Supplementary Information S1c). AR (Supplementary Information S1c) and cytokeratin 18 (CK-18) (data not shown) were expressed throughout the luminal epithelium of the normal prostate and decreased in the PIN epithelium.

The stroma was thickened around mPIN lesions with strong co-expression of α -smooth muscle (SM)-actin (Supplementary Information S1c) and vimentin (data not shown) consistent with a reactive myofibroblastic phenotype. An inflammatory response composed of mononuclear cells consistent with macrophages and lymphocytes was observed in 25% of the PPAR γ KO prostates, whereas it is not commonly noted in WT mouse prostates.¹⁸

Histologically, limited hyperplasia was found in the AP and DP of WT mice from 3 months. More widespread hyperplasia was seen in the AP and VP of PPAR γ KO mice from early stages. mPIN was observed in the AP and VP of PPAR γ KO mice (Figures 1e-h). The scores given in Figures 1e-h represent incidence of disease. When extent of disease was examined it was noted that the individual foci were markedly larger in older versus younger mice. Samples were scored by the presence of the most severe phenotype irrespective of its prevalence. mPIN was not seen in WT mouse prostates. In marked contrast, a 36.4% incidence of PIN (27.3% LGPIN plus 9.1% HGPIN) was observed in the AP of PPAR γ KO prostate in \leq 6 months, 26.9% PIN (7.7% plus 19.2%) in 7-12 month animals and 25% PIN (10% plus 15%) in \geq 13 months old mice. 100% PIN (54.5% plus 45.5%) was found in the VP of PPAR γ KO prostates in \leq 6 months, 84.6% PIN (38.5% plus 46.1%) in 7-12 months and 60% PIN in (40% plus 20%) in \geq 13 months. Histopathologic characterization indicates that PPAR γ is a regulator of mouse prostate epithelial cell differentiation and that its loss results in generally progressive mPIN.

Establishment of PPAR γ - and PPAR γ 2-deficient mouse prostate epithelial cell lines.

Two PPAR γ - and PPAR γ 2-deficient cell lines were developed. A spontaneously immortalized line was generated from the prostatic epithelium of a KO mouse. These mPrE-PPAR γ KO (mPrE- γ KO) cells incorporate complete functional deletion of PPAR γ , confirmed by PCR analysis (Figure 2a). A human U6-driven mouse PPAR γ 2 shRNA retroviral construct was introduced into a WT mouse prostatic epithelial cell line (mPrE).²⁰ These cells are designated mPrE-PPAR γ 2 shRNA (mPrE- γ 2sh). Western blot analysis demonstrated that the mPrE- γ KO cells have no PPAR γ 1 and γ 2 protein expression while the PPAR γ 2 shRNA construct was effective at reducing PPAR γ 2 protein expression (Figure 2b). The pSIR empty vector was used to make control cells (mPrE-pSIR).

mPrE- γ KO and mPrE- γ 2sh cells appeared similar, the cells were small and elongated with enlarged nuclei (Figures 2c and e). The PPAR γ - and PPAR γ 2-deficient cells grew as discreet individual cells with reduced cell-cell contact and with increased viability and proliferation (Figures 2d and f). Cell cycle analysis also showed fewer cells in S phase with mild increase in G0/G1 arrest and essentially no change in G2/M (Supplementary Table 1). mPrE- γ KO and mPrE- γ 2sh cells formed notably larger clones in a clonogenicity assay, than either mPrE or mPrE-pSIR cells (Figure 2g). Suppression of PPAR γ activity was confirmed using a luciferase reporter driven by a triple AOX-PPRE²¹ (Figure 2h). PPAR γ protein was undetectable by immunofluorescence (IF) staining in the nuclei of mPrE- γ KO and mPrE- γ 2sh cells (Figure 3a). p63 and CK-14 proteins were used to identify basal epithelial cells.¹⁸ But, p63 was weak to absent and cytokeratin (CK-14) protein expression was weaker in PPAR γ -/ γ 2-deficient cells *in vitro* (Figure 3a and Supplementary Information S2a). β -catenin protein expression was lost in the cellular membrane of growing colonies but was detected in the nuclei (Figure 3a). Western blot analysis demonstrated that p63, CK-14, β -catenin and E-cadherin proteins decreased in mPrE- γ KO

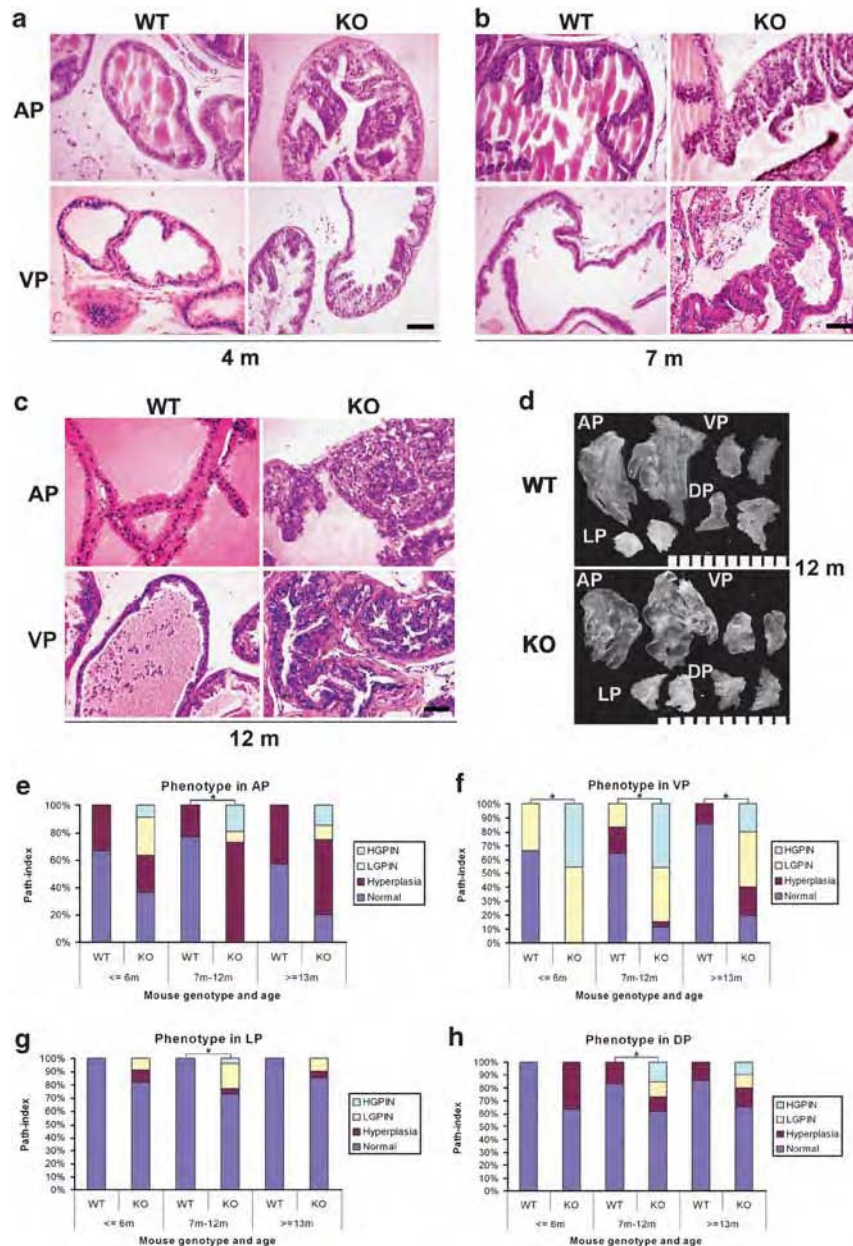


Figure 1 Progressive mouse prostatic intraepithelial neoplasia (mPIN) in conditional PPAR γ KO mouse prostates. (a, b and c) H&E stained sections illustrate the progressive development of mPIN from a low grade at the age of 3 months (a) to a high grade at the age of 7 months (b) and 12 months (c) in the AP and VP of PPAR γ KO mice prostatic epithelium, compared to a paired WT control mouse. Scale bar = 100 μ m in the panels. (d) Gross appearance of the four mouse prostate lobes, anterior (AP), ventral (VP), lateral (LP) and dorsal prostate (DP), dissected from 12 month old WT and conditional PPAR γ KO mice, illustrating similar gross appearance. Scale bar = 1 mm between two small bars. (e, f, g and h) Summary of the incidence of high grade PIN (HGPIN), low grade PIN (LGPIN), Hyperplasia and Normal in the AP, VP, LP and DP of age matched groups of WT and PPAR γ KO mouse groups at the ages of \leq 6 months (3 WT, 11 KO), 7-12 months (48 WT, 26 KO) and \geq 13 months (7 WT, 20 KO), Total 58 WT mice and 57 KO. * P < 0.01 by Fisher's Exact Test

and mPre- γ 2sh cells compared to mPre and mPre-pSIR controls (Figure 3b).

PPAR γ - and PPAR γ 2-suppression in a prostatic tissue recombination model resulted in mPIN pathogenesis.

To determine the consequences of PPAR γ - and PPAR γ 2-suppression in mouse epithelial cells *in vivo* with physiologically relevant epithelial-stromal interactions, we used a tissue recombination model. Recombinants

composed of untransfected mPre cells or control mPre-pSIR recombined with rat UGM formed glandular structures lined with cuboidal to columnar secretory epithelium surrounded by stroma resembling normal rodent prostate, consistent with previous observations²⁰ (Figures 3c-f). Tissue recombinants using either mPre- γ KO or mPre- γ 2sh cells exhibited similar mPIN phenotypes within two months of grafting (Figures 3c-f). Mild degrees of epithelial stratification, mild nuclear alterations, and reduced secretory

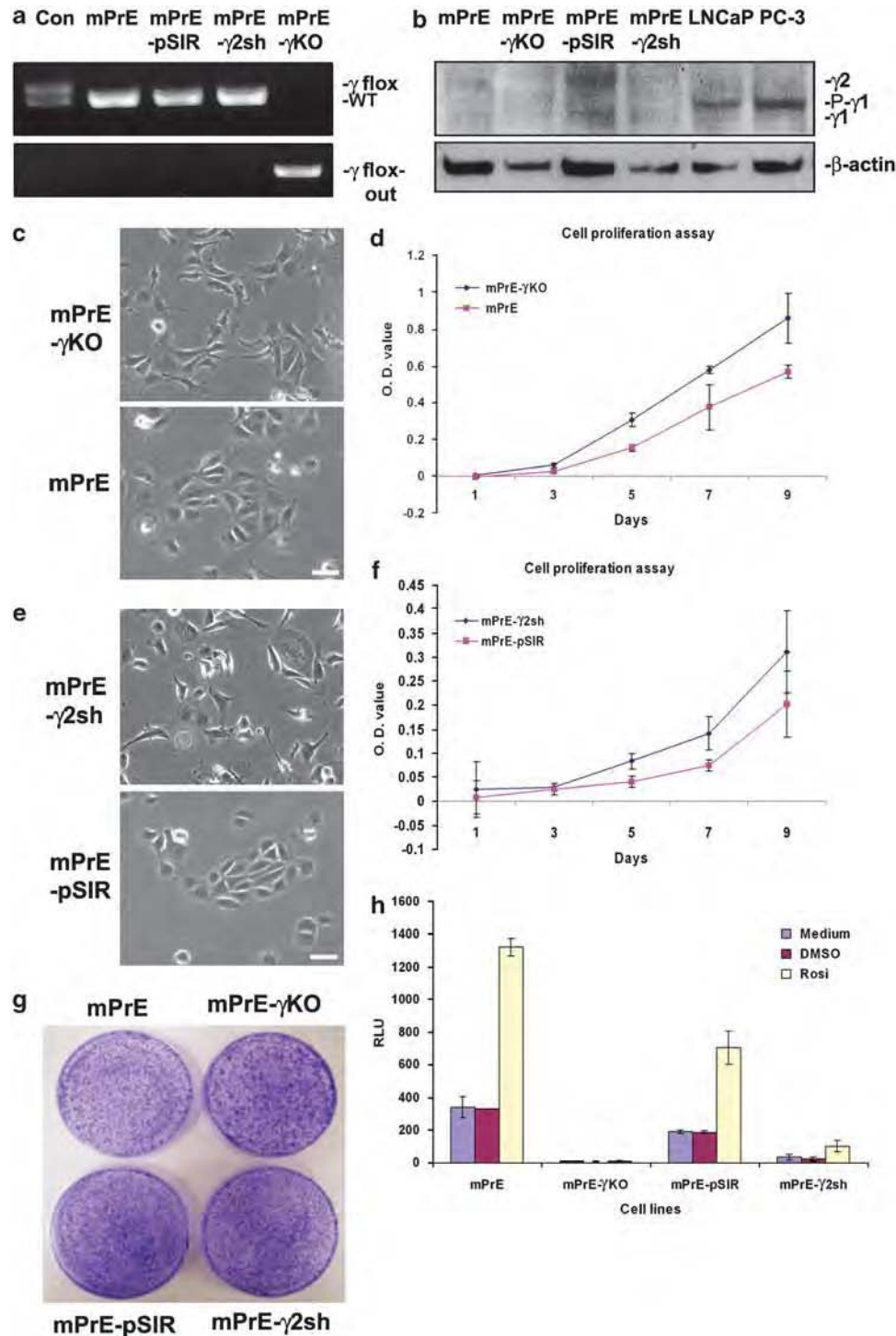


Figure 2 Establishment and characterization of the stable mPrE-PPAR γ knockout and mPrE-PPAR γ 2 knockdown cell lines. (a) Demonstration of genomic DNA for PPAR γ alleles in control mouse tail (Con, PBCre4^{0/0}PPAR γ ^{floxWT}), mPrE, mPrE-pSIR and mPrE- γ 2sh cell lines and its deletion (upper panel) and concurrent presence of a PPAR γ ^{flox-out} band (lower panel) in the mPrE-KO cell lines by PCR. (b) Western blot showing loss of PPAR γ 1 (γ 1) and PPAR γ 2 (γ 2) proteins expression in mPrE- γ KO cells and reduced PPAR γ 2 expression in mPrE- γ 2sh cells as compared to mPrE and mPrE-pSIR control cells. A Phosphorylated-PPAR γ 1 (P- γ 1) band showed only in LNCaP and PC-3 cells. (c and d) The effect of loss of PPAR γ on cellular morphology and proliferation. Phenotypically the control mPrE cells showed cobblestone morphology while mPrE- γ KO cells exhibited a more extended spindle-like phenotype with loss of cell-cell contacts. Scale bar = 50 μ m. Loss of PPAR γ resulted in increased cellular proliferation as determined by MTT assay. (e and f) The cellular morphology and proliferation of mPrE- γ 2sh and mPrE-pSIR cells in culture. Phenotypically these cells showed changes similar to those evoked by the knockout of PPAR γ in the mPrE- γ KO cells. Suppression of PPAR γ 2 protein expression resulted in increased cellular proliferation. (g) mPrE- γ KO and mPrE- γ 2sh cells form larger clones compared to control mPrE and mPrE-pSIR cells when tested using a clonogenicity assay. (h) PPRE activity detected by a luciferase reporter showed a > 85% decrease in the mPrE- γ 2sh cells as compared to mPrE controls. Signal was undetectable in mPrE- γ KO cells. Rosiglitazone has no effect on PPRE activity of mPrE- γ KO cells. However it shows mild activation on PPRE activity of mPrE- γ 2sh cells, confirming that these cells retain a weakened ability to respond to this PPAR γ agonist

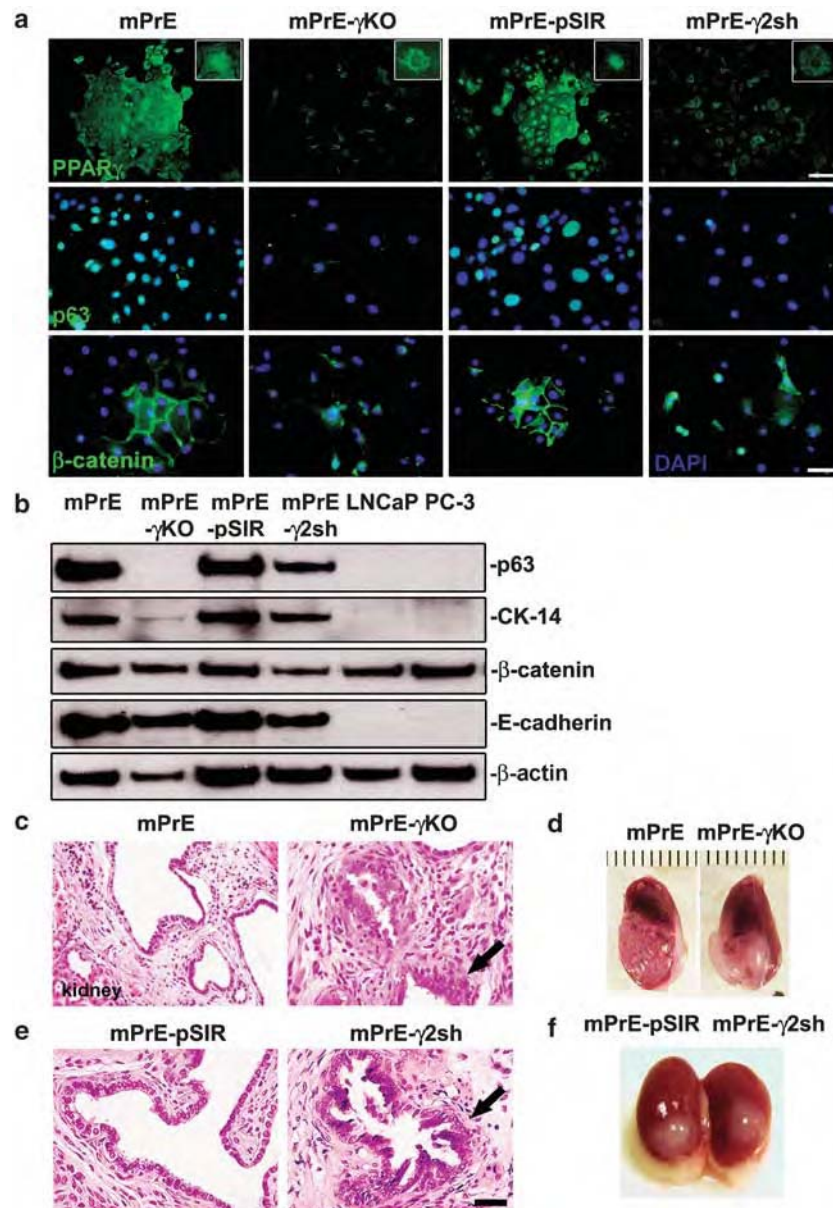


Figure 3 Consequences of PPAR γ deletion and suppression in prostate epithelial cells. (a) PPAR γ , p63 and β -catenin proteins detected by immunofluorescence in mPrE, mPrE- γ KO, mPrE-pSIR and mPrE- γ 2sh cells as compared to the control cells confirming the efficiency of disruption of PPAR γ protein expression (higher magnification in the inset). Loss of PPAR γ is associated with a loss of cobblestone morphology, cell-cell contacts, and an associated decrease in nuclear localization of p63 protein and membranous localization of β -catenin as well as nuclear transfer of β -catenin. Scale bar (PPAR γ) = 100 μ m. Scale bar (p63 and β -catenin) = 50 μ m. (b) Western blot analysis demonstrated that p63, CK-14, β -catenin and E-cadherin proteins decreased in mPrE- γ KO and mPrE- γ 2sh cells compared to mPrE and mPrE-pSIR controls. LNCaP and PC-3 cells were used as the controls. (c and e) Histology of tissue recombinants using rat urogenital sinus mesenchyme (UGM) with mPrE, mPrE- γ KO, mPrE-pSIR and mPrE- γ 2sh cells examined at two months post-grafting. The control recombinants resembled prostatic glandular differentiation although with some flattening of epithelial layers consistent with previous descriptions. Tissue recombinants made using mPrE- γ KO (c) and mPrE- γ 2sh (e) cells grew less readily. Histopathologically these structures exhibited a phenotype consistent with mPIN with epithelial crowding and tufting and out-growths (arrow). Scale bar = 50 μ m. (d and f) Gross appearance of tissue recombinants using mPrE and mPrE-pSIR cells were similar, showing glandular differentiation by two months post grafting. In contrast tissue recombinants made using mPrE- γ KO (d) cells and mPrE- γ 2sh (f) cells grew less readily and exhibited a less obviously glandular phenotype

differentiation were detected. These results parallel the mPIN prostate lesions of PBCre4^{tg/0}/PPAR γ ^{flox/flox} mice. Reduced luminal secretions were noted in mPrE- γ 2sh recombinants versus the controls, further suggesting reduced secretory differentiation (Figure 3). Of note, focal disruption of the basal membrane of reconstructed mPrE- γ KO mouse prostate

glands was seen on both H&E and Periodic acid Schiff (PAS) stained sections, and in some cases a few epithelial cells had migrated locally from the basal cell layer into surrounding stroma (Figures 3c), reminiscent of microinvasive carcinoma as defined in intact GEM prostates.¹⁸

Autophagocytosis in PPAR γ knockout and PPAR γ 2 knockdown mouse prostate epithelial cells. Ultrastructural analysis was performed on AP tissues from 12-month-old conditional PPAR γ KO and WT mice. WT AP is illustrated (Figure 4a). Foci of mPIN PPAR γ KO mouse were examined by electron microscopy. The secretory luminal cells of WT prostate were cylindrical with supra-nuclear

vesicles including some budding from the rough endoplasmic reticulum (RER) (Figures 4b-c). Number, size and distribution of mitochondria, lysosomes and peroxisomes were unremarkable. Secretory vesicles were found budding from the Golgi, and mature and exocytosing secretory vesicles were also observed. In contrast, the PPAR γ KO luminal epithelial cells in mPIN regions had a number of

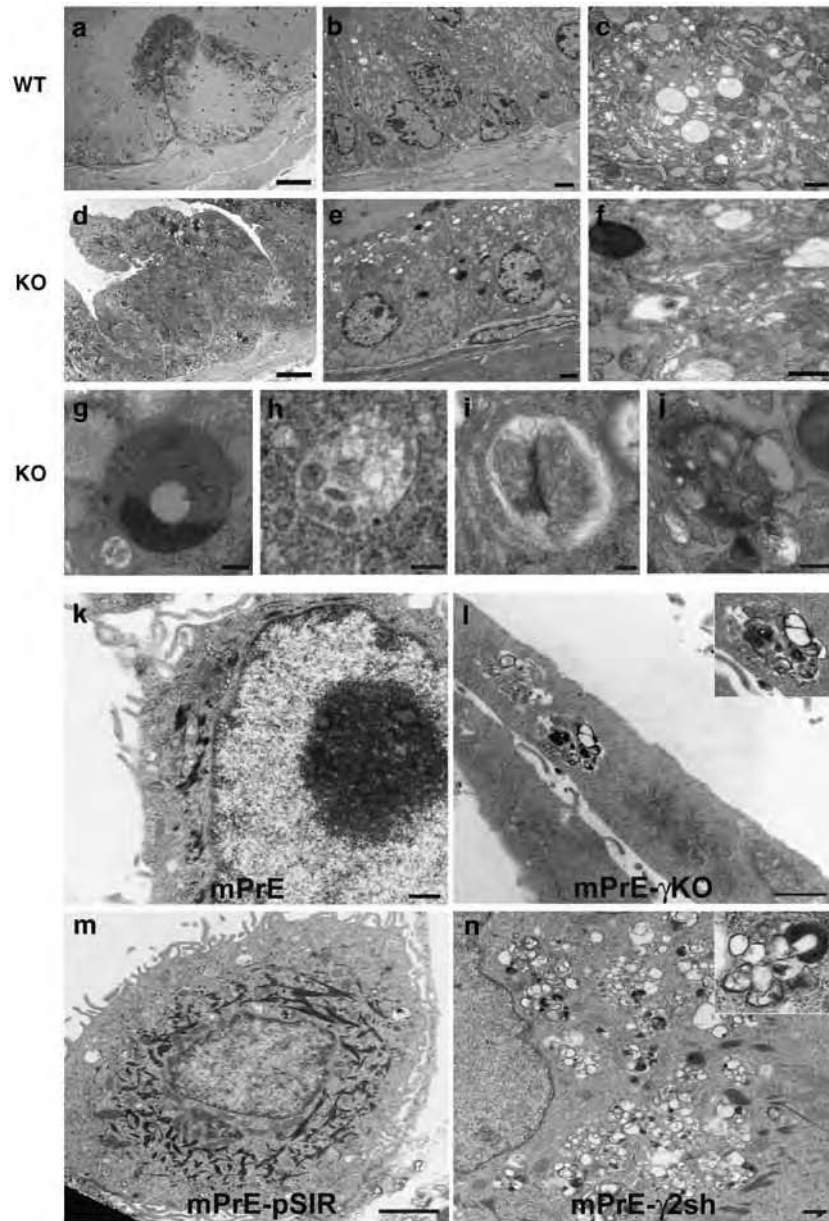


Figure 4 Increased autophagic features in the ultrastructure of PPAR γ knockout prostate epithelium and mPrE-PPAR γ 2 shRNA cells. Light and electron microscopy of prostate tissue indicated significant morphological differences between WT (a-c) and PPAR γ KO (d-f) mice at the age of 12 months. Toluidine blue staining of thick plastic sections (a and d) indicated increased cellularity and fewer secretory vesicles in the epithelium from PPAR γ KO mice prostate. Scale bar (a and d) = 50 μ m. Ultrastructurally, the WT mouse prostate epithelial cells showed a normal distribution of cytoplasmic constituents, organelles and secretory vesicles (b and c). In contrast, the PPAR γ KO cells had abnormal appearing mitochondria and increased numbers of lysosomes. The lysosomes had varied morphology. Some exhibited a classical appearance (e and f) or appeared as multivesicular bodies (f) while others had the appearance of autophagosomes (g-j). Scale bars: (b) = 2 μ m, (c) = 500 nm, (e) = 2 μ m, (f) = 500 nm, (g) = 500 nm, (h) = 100 nm, (i) = 100 nm and (j) = 500 nm. Cytoplasmic organelles in mPrE and mPrE-pSIR cells had a normal distribution and ultrastructural appearance (k and m). In contrast mPrE- γ KO cell demonstrated a decrease in mitochondria and increase in secondary lysosomes (l). Inset: increase magnification image lysosome containing numerous membranes and other debris, suggestive of autophagosome. mPrE- γ 2sh cells had increased lysosomes, many having the appearance of autophagosomes (n). Inset: autophagosome. Scale bars (k) = 500 nm, (l) = 1 μ m, (m) = 2 μ m and (n) = 500 nm

abnormal features. There were enlarged nuclei with large nucleoli and few clear storage vesicles were seen in the cytoplasm. Many of the mitochondria appeared to be degenerating and there were 2.4-fold more lysosomes in the HGPIN compared to WT epithelium (Figures 4e-f). The lysosomes varied in size and many showed features suggestive of autophagocytosis, including both early and late autophagosomes.²² The PPAR γ KO epithelial cells in mPIN foci contained clusters of variably sized autophagic vacuoles (autophagosomes) containing loose granular to more dense flocculent substance. Features suggesting autophagocytosis included double membranes, internal vesicles, and the presence of organelles such as mitochondria, peroxisomes, and rough endoplasmic reticulum within the lysosome (Figures 4f-j). There were also a number of multivesicular bodies (Figure 4f). Fewer normal-appearing secretory vesicles were present (Figures 4e-f). In some places, the basement membrane was disrupted at the site of basal cell attachment (Figure 4e). These changes were consistent with a reduction in secretory differentiation of prostatic luminal cells and possible increase in autophagy in the proliferating mPIN regions.

mPrE-pSIR and mPrE cells exhibited normal number, size and distribution of cellular organelles and secretory vesicles (Figures 4k and m). In contrast, the mPrE- γ KO and mPrE- γ 2sh cells had a substantially increased range of lysosomal sizes where the majority were small and distributed as clusters within the cytoplasm. Many of these were complex with lipid whorls and internalized material, including mitochondria, peroxisomes and rough endoplasmic reticulum, in various stages of digestion (Figures 4l and n). Thus, while the changes were more dramatic in the mPrE- γ 2sh cells, the overall ultrastructural alterations were reminiscent of the cytoplasmic changes noted in the prostatic epithelium from PPAR γ KO mice and were consistent with increased autophagocytosis (autophagy).

Gene expression profiles in the mPrE- γ KO cells vs. mPrE cells or mPrE- γ 2sh cells vs. mPrE-pSIR cells demonstrated major changes (Table 1) including alterations in signaling pathways involving genes of the nuclear receptor II subfamily, cell cycle control (dedifferentiation), peroxisomal and mitochondrial lipid transporter and oxidation metabolism (oxidative stress/hypoxia) and ubiquitination/proteosomal degradation. Ingenuity Pathway Analysis (IPA, Ingenuity Systems) was used to evaluate changes based on microarray data. The top canonic pathway and network related to PPAR γ -signaling signatures were metabolism pathway/oxidative stress (Supplementary Information S3a-b) and cell cycle control/dedifferentiation (Supplementary Information S4a-b). Effects were generally consistent between PPAR γ KO and PPAR γ 2 shRNA prostate epithelial cells as compared to control cells.

Altered expression patterns of autophagy-associated proteins were detected in mPrE- γ KO and mPrE- γ 2sh cells compared to the control cells (Figure 5a). LC-3 (Atg8), beclin-1 (Atg6), activated caspase-3, PCNA (Figure 5a), and COX-2 and vimentin (Supplementary Information S2a) were increased, whereas catalase (Figure 5a) and PMP70 (data not shown) were decreased in two PPAR γ -/ γ 2-deficient cell lines.

Monodansylcadaverine (MDC), a marker of autophagy,²² was visualized in the mPrE and mPrE- γ KO, mPrE-pSIR and mPrE- γ 2sh cells under 5% FBS (regular growth) or 2.5% FBS (semi-starvation) culture conditions. Elevated MDC signaling was seen in both mPrE- γ KO and mPrE- γ 2sh as compared to control cells. This was enhanced in 2.5% FBS culture medium (Figure 5b). Lysosomes and mitochondria were tracked, using LysoTracker (DND-99) and MitoTracker,²² respectively, under the 5% or 2.5% FBS culture conditions. The LysoTracker (Supplementary Information S2b) and MitoTracker (data not shown) signaling were strongly increased in PPAR γ -/ γ 2-deficient cell lines in 2.5% FBS culture medium.

In vivo, catalase (Figure 5c) and PMP70 (data not shown) proteins were found to be decreased or lost in mPIN foci of KO mice. In contrast, LC-3 protein (Figure 5c) was increased in the mPIN foci of KO mice compared to the epithelium of WT mice.

Active PPAR γ 1/ γ 2 signaling suppresses the phenotypes in mPrE-PPAR γ KO cells and mPrE-PPAR γ 2 knockdown tissue recombinants.

To dissect the biological functions of PPAR γ 1 and PPAR γ 2 isoforms we re-expressed PPAR γ 1 or PPAR γ 2 cDNA into mPrE- γ KO cells viral transduction of wild-type full-length cDNA.²³ Three cell lines, mPrE-PPAR γ KO-empty vector (mPrE- γ KO-EV), mPrE-PPAR γ KO-PPAR γ 1 WT (mPrE- γ KO- γ 1WT) and mPrE-PPAR γ KO-PPAR γ 2 WT (mPrE- γ KO- γ 2WT), were established (Figure 6a). Western blotting confirmed re-expression of the PPAR γ 1 or PPAR γ 2 isoform (Figure 6b). IF staining showed positive nuclear PPAR γ protein in mPrE- γ KO- γ 1WT and mPrE- γ KO- γ 2WT cells compared to the control mPrE- γ KO-EV cells (Figure 6a). While total β -catenin levels were not greatly altered there was a marked shift in localization from the nuclei in the KO cells to the cytoplasm and membrane in the PPAR γ 1-/ γ 2-expressing cells (Figure 6a). Elevated levels of E-cadherin (Figure 6b), but not CK-14 and p63 proteins (data not shown), were seen in mPrE- γ KO- γ 1WT and mPrE- γ KO- γ 2WT cells. EM showed decreased lysosome and autophagosome volume and increased numbers of mitochondria in the PPAR γ 1-/ γ 2-rescued mPrE- γ KO cells compared to the control mPrE- γ KO-EV cells (Figure 6c). mPrE- γ KO- γ 1WT and mPrE- γ KO- γ 2WT cells showed decreased cell viability and proliferation (Supplementary Information S5a) as well as clonal formation (Supplementary Information S5b). As expected mPrE- γ KO- γ 1WT and mPrE- γ KO- γ 2WT cells showed increased PPARE-activity by a luciferase reporter assay (Supplementary Information S5c). IF staining showed increased levels of CK-14 and catalase (Supplementary Information S5d), decreased levels of caspase-3 activation (Figure 6a), LC-3 and PCNA (Supplementary Information S5c), and beclin-1 (data not show) in the PPAR γ 1-/ γ 2-rescued cells compared to the mPrE- γ KO-EV control cells.

Tissue recombinants of mPrE- γ 2sh cells showed decreased secretion and immunophenotypic features of PIN phenotypes consistent with PPAR γ KO mouse prostates. These included reduction in p63 positive basal cells and AR (Figure 6d) with lesion progression and phenotypic alterations in stromal cells visualized by α -SM-actin staining (Figure 6d). Basal cells progressively decreased as the mPIN lesion

Table 1 Major gene expression profiling changes identified from microarray in mPrE- γ KO cells vs mPrE cells or mPrE- γ 2sh cells vs mPrE-pSIR cells (> 2 fold and < 2 fold)

Description	Systematic	Official symbol	mPrE- γ 2sh	mPrE- γ KO
1. Sub-cellular organelles/Lipid metabolism				
Catalase	vm059759	Cat	0.45	0.12
Prostaglandin-endoperoxide synthase2(COX-2)	vm076500	Ptgs2	6.10	45.3
Isovaleryl CoA dehydrogenase	vm073968	Ivd	0.40	0.01
Peroxisomal biogenesis factor 6	vm081528	Pex6	2.07	21.3
Peroxisomal acyl-CoA thioesterase 1 (Pte1)	vm085581	Acot8	2.05	13.18
Hydroxy-delta-5-steroid dehydrogenase	vm081035	Hsd3b7	8.02	2.42
Solute carrier family 27 (fatty acid transporter)	vm062392	Slc27a3	10.34	5.30
ATP-binding cassette, sub-family A, member 3	vm060761	Abca3	3.82	321.9
Sterol carrier protein 2, liver	vm084113	Scp2	2.01	16.2
Oxysterol binding protein	vm079937	Osbp	71.6	18.5
Acetyl-CoA acyltransferase 1 (peroxisomal)	vm083546	Acaa1	0.42	0.44
Mitochondrial NADH dehydrogenase 5	vm083510	Ndufa5	3.20	2.16
Acetyl-CoA acyltransferase 2 (mitochondrial)	vm088089	Acaa2	0.45	0.15
ATP synthase, H+ transporting, mitochondrial F 1	vm068155	Atp5o	0.44	0.38
ATP synthase, H+ transporting, V1 subunit D	vm083645	Atp6v1d	0.41	0.37
Cytochrome P450, family 26, subfamily b	vm064755	Cyb26a1	0.01	0.01
3-hydroxybutyrate dehydrogenase	vm064957	Bdh1	0.50	0.25
Lysosomal mannosidase, beta A	vm071702	Manba	2.11	16.80
Lysosomal-associated protein transmembrane 4A	vm060108	Laptm4a	0.50	0.33
Vesicle-associated membrane protein 5	vm070176	Vamp5	0.20	0.13
Vesicle-associated membrane protein 8	vm068808	Vamp8	0.43	0.37
2. Oxidative stress (Hypoxia)				
Hypoxia up-regulated 1	vm062588	Hyou1	2.17	21.2
Hairless	vm086899	Hr	2.62	76.70
Forkhead box O 6	vm078799	Foxo6	2.13	2.77
Oxidation resistance 1	vm077473	Oxr1	0.29	0.35
Hairy and enhancer of split 1	vm058417	Hes1	0.5	0.28
Microsomal glutathione S-transferase 1	vm059398	Mgst1	0.01	0.09
3. Cell cycle control (de-differentiation and EMT)				
Proliferation cell nuclear antigen	vm072868	Pcna	2.03	28.5
Ki67	vm078634	Mki67	2.22	13.2
Cell cycle progression 1	vm085838	Ccpg1	2.18	120.2
Cyclin G associated kinase	vm085772	Gak	2.45	44.8
Cyclin-dependent kinase 2	vm087446	Cdk2	2.08	44.3
Vimentin	vm087332	Vim	775.70	738.70
4. Nuclear receptors/cofactors subfamily				
Peroxisome proliferative activated receptor, gamma, coactivator 1beta	vm078414	Ppargc1b	0.01	0.01
Vitamin D receptor	vm076701	Vdr	0.01	0.01
Cellular retinoic acid binding protein II	vm057044	Crabp2	0.01	0.01
cAMP responsive element binding protein 1	vm086761	Creb1	0.01	0.01

Global gene expression profiling in mPrE-PPAR γ knockout cells vs mPrE cells or mPrE-PPAR γ 2 knockdown cells vs mPrE-pSIR cells. The major signaling pathway changes in mPrE- γ KO cells vs mPrE cells and mPrE- γ 2sh cells vs mPrE-pSIR cells by microarray analysis were involved in metabolic lipid oxidation, subcellular organelles/autophagy, oxidative stress, cell cycle regulation/cellular dedifferentiation and nuclear receptors/PPRE. Expression value of an individual gene was by the comparison of mPrE- γ KO cells vs mPrE cells or mPrE- γ 2sh cells vs mPrE-pSIR control cells.

progressed, similar to the pattern recognized in human PIN progression and in progressively severe mPIN in some GEM models.¹⁸ To examine the potential for targeted chemoprevention with pharmacologic PPAR γ agonists, tissue recombinants composed of mPrE- γ 2sh cells plus rat UGM were grown for three months in animals which were continuously fed on chow containing the PPAR γ agonist Rosiglitazone. At sacrifice the grafts in these animals were of similar size to mPrE-pSIR controls and on histologic examination, secretory differentiation of luminal epithelial cells and intraluminal secretions were increased compared to mPrE- γ 2sh recombinants without drug treatment. mPIN-like changes were reduced compared to grafts of control mice fed on standard animal chow. Rosiglitazone treatment enhanced p63 and AR expression and also modified the stromal compartment in mPrE- γ 2sh tissue recombinants (Figure 6d).

These data support the roles of reduced PPAR γ function in the development of mPIN and indicate that re-expression of either PPAR γ 1 or PPAR γ 2 isoform in PPAR γ KO cells is able to rescue the phenotype. Rosiglitazone treatment suppressed mPIN formation in mPrE- γ 2sh tissue recombinants, likely by activating transcription through PPAR γ 1, although PPAR γ -independent effects cannot be excluded.

Discussion

Elucidating the molecular pathologic mechanisms underlying prostatic carcinogenesis may allow for more conservative targeted medical treatments for certain low risk patients with precursor lesions or organ confined, small volume, low grade carcinomas. The ability to medically inhibit progression of HGPIN to Pca or the progression of small, lower grade Pcas

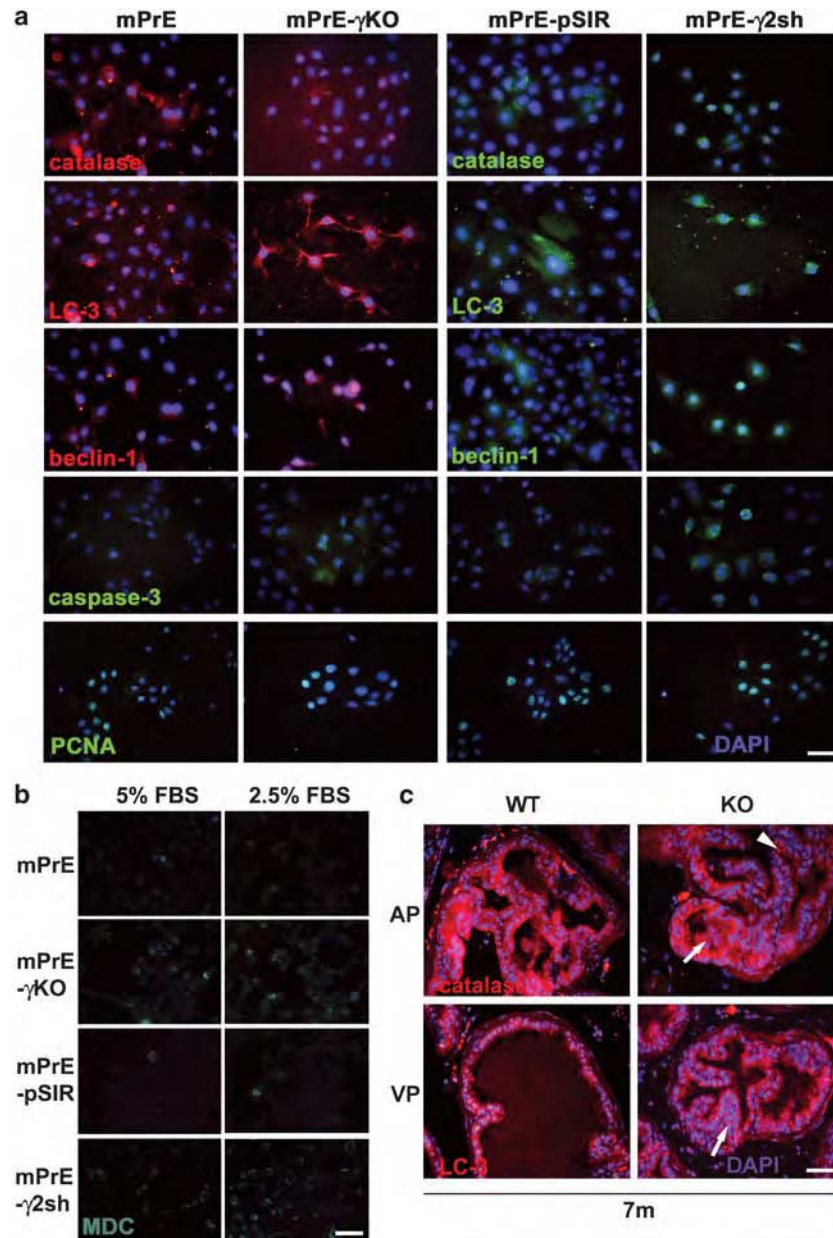


Figure 5 Alterations in autophagy-associated protein expression in mPrE-PPAR γ knockout and mPrE-PPAR γ 2 shRNA cells. (a) Catalase, LC-3 (Atg8), beclin-1 (Atg6), caspase-3 and PCNA proteins were detected by immunofluorescence staining in mPrE and mPrE- γ KO, mPrE-pSIR and mPrE- γ 2sh cells grown on glass slides for three days. Decreased catalase and increased LC-3 and beclin-1 were seen in mPrE- γ KO and mPrE- γ 2sh cells compared to mPrE and mPrE-pSIR cells. These results suggested active autophagic body formation in the cells. Meanwhile, caspase-3 and PCNA were increased in PPAR γ - γ 2-deficient cells. Scale bar = 50 μ m in the panels. (b) MDC, a marker of autophagy, was visualized in mPrE and mPrE- γ KO, mPrE-pSIR and mPrE- γ 2sh cells under the 5% FBS regular or 2.5% FBS half-starvation culture conditions. Elevated staining was seen in both mPrE- γ KO and mPrE- γ 2sh cells as compared to mPrE and mPrE-pSIR cells. The signals were strongly increased in PPAR γ - γ 2-deficient cells in the 2.5% FBS culture media. Scale bar = 50 μ m in the panels. (c) Immunofluorescence staining of catalase and LC-3 in wild-type (WT) and PPAR γ knockout (KO) prostate tissue at ages of 7 months. Low levels of catalase expression and high expression of LC-3 protein were seen in the PIN regions (arrows) of PPAR γ KO mouse prostate epithelium compared to WT and more normal-appearing areas (arrowhead). Scale bar = 50 μ m in the panels

to larger, higher grade tumors would represent a significant advance for Pca patient management.

We propose that PPAR γ is a key regulator in the maintenance of peroxisomal, mitochondrial and lysosomal functions. Genetic disruption of PPAR γ or PPAR γ 2 signaling in mouse prostate epithelial cells resulted in dysregulated expression patterns of peroxisomal and mitochondrial genes

whose products are involved in lipid transportation and oxidation pathways. Active autophagosomes and abnormally increased numbers of lysosomes were found in PPAR γ - and PPAR γ 2- deficient prostatic epithelia. *In vitro* these phenotypes were rescued by re-expression of PPAR γ 1 and PPAR γ 2 isoform in mPrE-PPAR γ KO cells. *In vivo* changes consequent to loss of PPAR γ were associated with hyperplasia, PIN

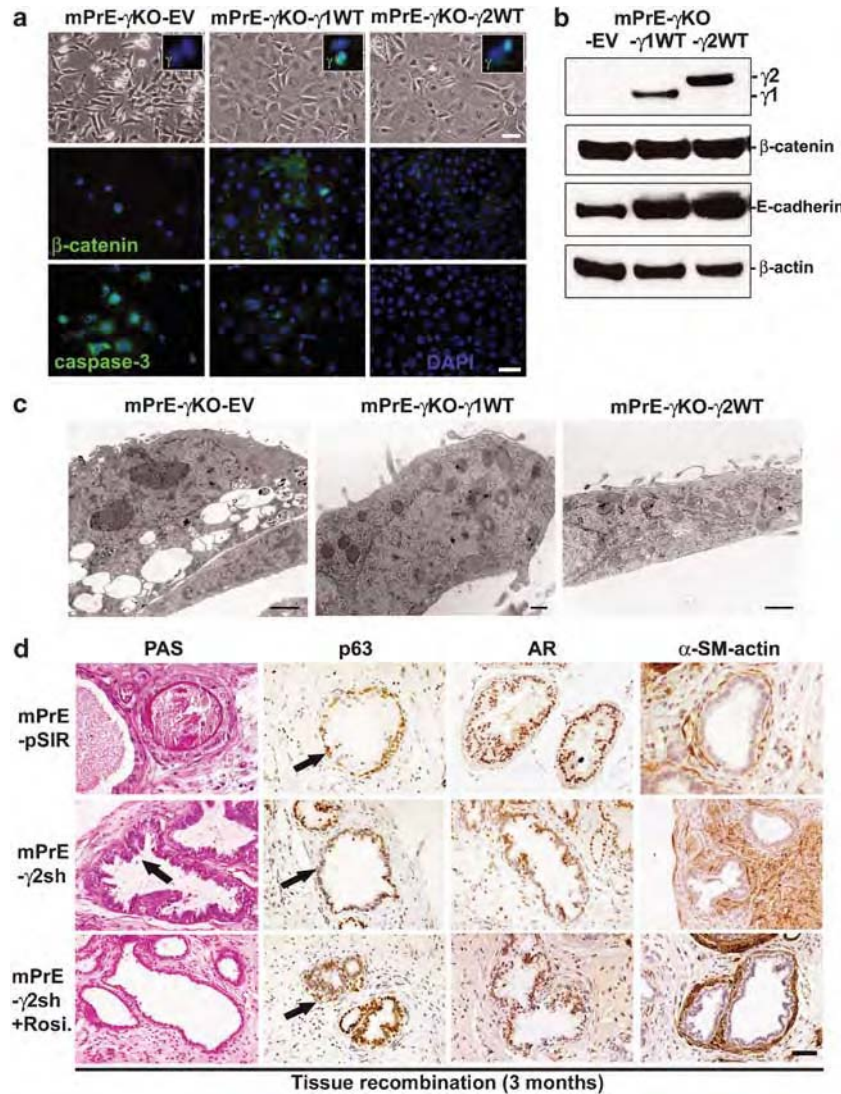


Figure 6 Rescue of phenotypes of mouse PPAR γ -deficient prostate epithelial cells by re-expression of either PPAR γ 1 or PPAR γ 2 isoform or treatment by Rosiglitazone. (a) Phase-contrast microscopy morphology of control mPrE- γ KO-empty vector and mPrE- γ KO-PPAR γ 1 or -PPAR γ 2 WT cDNA cells, showing a return to a cobblestone morphology following reintroduction of each PPAR γ isoform. IF staining confirmed expression of PPAR γ protein in the nuclei of mPrE- γ KO- γ 1WT and mPrE- γ KO- γ 2WT cells (inset boxes). β -catenin protein was predominantly nuclear in mPrE- γ KO cells, whereas in mPrE- γ KO- γ 1WT and mPrE- γ KO- γ 2WT cells the protein was found in the cytoplasm and on intercellular membrane interfaces. Caspase-3 was decreased in mPrE- γ KO- γ 1WT and mPrE- γ KO- γ 2WT cells compared to mPrE- γ KO-EV cells. Scale bar = 50 μ m. (b) Western blot analysis demonstrated PPAR γ 1 protein in mPrE- γ KO- γ 1WT and PPAR γ 2 in mPrE- γ KO- γ 2WT cells. β -catenin and E-cadherin protein levels increased in mPrE- γ KO- γ 1WT and mPrE- γ KO- γ 2WT cells compared to mPrE- γ KO-EV control cells. (c) mPrE- γ KO-EV cells had decreased mitochondria and increased lipid droplets and secondary lysosomes, similar to mPrE- γ KO cells. Scale bar = 2 μ m. However introduction of the mouse PPAR γ 1 wild-type cDNA increased mitochondria above wild-type values and decreased the presence of secondary lysosomes and lipid accumulation. Scale bar = 250 nm. Likewise, introduction of the mouse PPAR γ 2 wild-type cDNA into mPrE- γ KO cells restored the level of mitochondria and reduced the instances of secondary lysosomes and lipid droplets. Scale bar = 500 nm. (d) Tissue recombinants made using control (mPrE-pSIR) or mPrE- γ 2sh cells with rat UGM. Sections were examined for secretion by PAS. In control recombinants a normal prostatic phenotype with secretion was noted. In contrast in the mPrE- γ 2sh containing recombinants a low grade mPIN (arrow) with less secretion into the luminal space and thickened stroma was seen. p63 and AR protein were decreased in the mPrE- γ 2sh containing recombinants, but α -SM-actin protein expression was increased compared to tissue recombinants of mPrE-pSIR. Mice carrying tissue recombinants made by mPrE-pSIR and mPrE- γ 2sh cells were administered Rosiglitazone chow (0.005% Rosiglitazone) from the time of grafting until sacrifice at three months. Tissue recombinants of mPrE- γ 2sh in these mice (designated mPrE- γ 2sh + Rosi.) showed secretion, and increased p63 protein in the basal layer and p63 negative-luminal differentiation by IHC staining (arrows). These recombinants showed well-differentiated prostatic glandular structure and a more normal-appearing stroma. Scale bar = 50 μ m in the panels

formation and progression to malignancy, which in the case of PPAR γ 2 suppression could be rescued using high levels of the PPAR γ agonist Rosiglitazone. The pathophysiological roles of PPAR γ in cellular peroxisomal and mitochondrial fatty acid oxidation and autophagy based on results are summarized in Figure 7.

Alterations in lipid metabolism resulting in loss of PPAR γ -signaling have been suggested to predispose the prostate to premalignant or malignant changes.^{24–26} The current study demonstrates, for the first time, that PPAR γ loss-of-function in wild-type mouse prostatic epithelium results in progressive epithelial hyperplasia with atypia, indicative of mPIN. Using

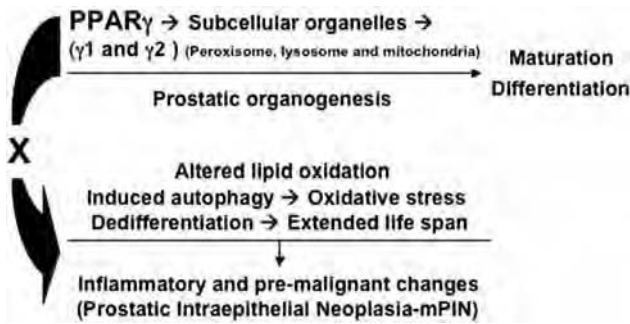


Figure 7 Disruption of PPAR γ resulted in mouse prostate carcinogenesis involving oxidative stress and autophagy. The simplified diagram brings together data from the studies and presents a model illustrating how the PPAR γ signaling contributes to prostate carcinogenesis from wild-type to mPIN formation and to set up conditions that would predispose cells to further malignant progression. These data suggested an important role for the PPAR γ gene in maintaining the maturation, differentiation and turnover of subcellular organelles (peroxisomes, mitochondria and lysosomes) during mouse prostatic organogenesis and development. In particular this model suggests that a mechanism by which loss of PPAR γ could lead to mouse PIN related to the disruption of cellular peroxisomal and mitochondrial lipid metabolism and oxidative stress (hypoxia) and active autophagy for the extended life span and cellular dedifferentiation

both intact mice with targeted knockout of PPAR γ in prostate epithelium and a tissue recombination model with selective gene modulation in the epithelial compartment reduced PPAR γ function was associated with increased activation of oxidative stress, autophagic activity, and activation of pro-inflammatory signaling pathways. This establishes conditions for subsequent malignant transformation which would be expected to occur stochastically, resulting from epithelial genomic damage potentially caused by reactive oxygen species (ROS).²⁶ The results described here parallel reported changes in gene expression resulting in reduced ligands for PPAR γ in the human prostate and provide the first direct evidence that loss of PPAR γ expression or function can lead to prostatic neoplasia *in vivo*.

Reduced activation of PPAR γ due to reduced formation of endogenous ligands for PPAR γ most likely explains its role early in human Pca development.^{8,27,28} Our studies demonstrate that PPAR γ plays a role in maintaining peroxisomal, mitochondrial and lysosomal biogenesis and maturation during prostatic epithelial cellular growth and differentiation. Catalase and PMP70 proteins were decreased in PPAR γ - and PPAR γ 2-deficient prostate epithelial cells. Degenerated mitochondria were found in two PPAR γ - and γ 2-deficient prostate epithelial cell lines. Active autophagosomes and abnormally increased numbers of lysosomes were found in PPAR γ - and PPAR γ 2-deficient prostatic epithelia. These changes were associated with mouse PIN formation and progression. Microarray analysis revealed alterations to peroxisomal and mitochondrial fatty acid transporter synthesis and oxidative metabolism in both PPAR γ - and γ 2-deficient mPrE cells as compared to the control cells. Disruption of PPAR γ -signaling results in altered fatty acid metabolism and induction of oxidative stress and hypoxia, as supported by the increased Hypoxia up-regulated 1 (Hyou1) and Hairless (Hr), and decreased Hairy and enhancer of split1 (Hes1) gene expression in addition COX-2, GSTs and uPAR

which show similar changes in expression patterns in clinical samples of human PIN and in other mouse models.^{29,30} A number of these altered genes contain a PPRE domain^{31,32} suggesting that these might be directly regulated. However, regulation of crucial genes could also occur indirectly, as a secondary consequence of reduced PPAR γ -signaling. The combination of light microscopic, ultrastructural, immunophenotypic, and gene expression data are consistent with dramatic changes in multiple subcellular organelles including mitochondria, peroxisomes and lysosomes, which likely contribute to the observed neoplastic phenotypes.

Our data suggest that the oxidative stress induced by PPAR γ loss-of-function results in lysosomal autophagy which can contribute to malignant progression.³³ Staining using lysosome tracker Red DNT-99 and autophagy markers MDC, LC-3 and beclin-1 showed increased signal strength in PPAR γ - and γ 2-deficient mPrE cells *in vitro* and *in vivo* confirming autophagosome formation induced by active lysosomes, consistent with electron microscopy showing lysosomal changes. Changes included increased lysosomal number with variable sized including many small structures, often accumulating in clusters in PPAR γ -deficient mPrE cells. This appears to be an abnormal autophagic response which does not result in cell death, possibly allowing individual cells to avoid an increase in apoptotic activity (indicated by increased caspase-3 activation) induced by loss of PPAR γ activity.

The role of autophagy in cancer is complex.³⁴ Autophagy may be pro-tumorigenic, promoting tumor cell survival and restricting necrosis.^{35,36} Alternatively autophagy could represent either a barrier, or an adaptive response, to cancer. Here, using both deletion (PPAR γ KO) and suppression (PPAR γ 2 shRNA) of PPAR γ in mouse prostate epithelial cells, we show increased autophagic activity. We suggest that PIN may indirectly result from the deregulation of pro-inflammatory pathways following PPAR γ inactivation combined with extension of life span via increased autophagic capacity. This outcome may be related to disruption of peroxisomal lipid oxidation/metabolism signaling pathways.

Early prostate cancer has been linked to a loss of enzymes including 15-lipoxygenase-2 (15-LOX-2) which is involved in the generation of 15(S)-hydroxyeicosatetraenoic acid (15-HETE).^{8,27} Such a scenario justifies the consideration of PPAR γ agonists as chemopreventive agents to inhibit the genesis of early stage prostate cancer. Based upon the data presented here we would suggest that modulation of PPAR γ signaling by glitazone drugs be considered as an addition to anti-oxidant diets to inhibit progression of HGPIN to prostate cancer.

Materials and Methods

Animal experiments. Floxed PPAR γ ³⁷ and PBCre4³⁸ transgenic mouse lines have been described previously. PBCre4 mice were backcrossed in C57/Bl6 for more than 10 generations. Double transgenic PBCre4^{tg/tg}/PPAR γ ^{fllox/fllox} mice were generated by breeding the PBCre4 and floxed PPAR γ transgenic lines and were maintained in a C57/Bl6 background. Wild-type littermates were used as the control groups. PCR primers for PPAR γ genotyping and for detecting DNA recombination and excision have been previously reported.³⁷ Adult male severe combined immunodeficient (SCID) mice [C.B.17/lcrHsd-scid] were purchased (Harlan, Indianapolis, IN). All work involving animals was performed under protocols reviewed and approved by the Vanderbilt IACUC.

H & E, immunofluorescence (IF) and immunohistochemical (IHC) staining. Mouse prostate lobes and tissue recombinants were dissected and fixed in 10% phosphate-buffered formalin overnight, transferred to 50% ethanol, then embedded in paraffin. Samples were sectioned for 8 successive layers at 5 μ m intervals and stained with hematoxylin and eosin (H&E). IF and IHC were performed as previously described.²⁷ The observation was under a fluorescence microscope (ZEISS, Axio imager M1) equipped with an appropriate filter system.

Electron microscopy (EM). Prostate tissue and fresh cell pellets were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1M, pH 7.2) overnight at 4 °C and washed in the same buffer for 30 min followed by post-fixation for 2 h at 4 °C in 1% osmium tetroxide (OsO₄) in cacodylate buffer. After fixation, the material was dehydrated through a graded series of alcohols and embedded in Spurr Resin. For light microscopic analysis, semi-thin sections (2 μ m) were stained with toluidine blue. Ultrathin sections (70 nm) were cut and ultrastructural analysis was performed on a Phillips CM-12 Transmission Electron Microscope operated at 80 KeV. Images were acquired using an AMT digital camera system. For quantification of lysosome number per area of cell cytoplasm examined, four separate thin sections were analyzed. The number of cross sections through lysosomes was counted and the area of cell cytoplasm represented in the four thin sections was determined. Given that most lysosomes are roughly spherical in shape, a lysosome would not be sampled twice in this procedure. Although, the exact number of lysosomes per cell cannot be obtained using this procedure, differences in the relative occurrence of lysosomes between wild type and PPAR γ -deficient cells can be estimated with reasonable accuracy.

Tissue recombinants and sub-renal capsule xenografting. Single cell suspensions of rat UGM were prepared from E18.5 embryonic fetuses as previously described.²⁰ Viable cells were counted using a hemacytometer. To prepare tissue recombinants, rat UGM was mixed with genetically modified mouse prostate epithelial cells at a ratio of 250 000 to 400 000. The cell mixture was pelleted and resuspended in 50 μ l of rat-tail collagen (pretitrated to pH 7.4). After polymerization, the collagen was overlaid with growth medium. After incubation at 37 °C overnight, the tissue recombinants were grafted under the renal capsule of intact CB17/cr/Hsd-SCID mouse. Hosts were sacrificed at 4 weeks, 8 weeks and 12 weeks post-grafting. The kidneys with the grafts were removed and imaged before processing for histology.

Administration of Rosiglitazone to mice. Host SCID mice were fed by prepared BRL-49653 (Avandia or Rosiglitazone) chow (0.005 % Rosiglitazone) RTD C056 (TestDiet, Richmond, IN)³⁹ for seven days before xenografting surgery. And then subsequently for three months until sacrifice. The control mice were fed matched regular rodent chow.

Statistical analysis. A Statistical Analysis System (version 9.1, SAS Institute, Cary, NC) was used. Pathological index of wild-type and PPAR γ knockout mice were divided into four groups of Normal, Hyperplasia, LGPIN and HGPIN. Percent of each index in each lobe, (AP, VP, LP or DP), at different ages; \leq 6 months, 7-12 months and \geq 13 months was analyzed by Fisher's Exact Test. Differences were considered statistically significant at $P < 0.05$.

Accession number. All microarray data have been deposited at the GEO database, accession number GSE13867 and a link for review:

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=dbgfmkkaieiu-np&acc=GSE13867>

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